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AUTHOR(S):

Kanamori, Yohei; Murakami, Masaru; Matsui, Tohru; Funaba, Masayuki

CITATION:

Kanamori, Yohei ...[et al]. The regulation of hepcidin expression by serum treatment: requirements of the BMP response element and STAT- and AP-1-binding sites.. Gene 2014, 551(2): 119-126

ISSUE DATE:

2014-11-10

URL:

http://hdl.handle.net/2433/191204

The regulation of hepcidin expression by serum treatment: requirements of the BMP response element and STAT- and AP-1-binding sites

Yohei Kanamori[1], Masaru Murakami[2], Tohru Matsui[1] and Masayuki Funaba[1]*

[1]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

[2]Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara 252-5201, Japan

Running title: Serum-induced hepcidin expression

*Corresponding author: Masayuki Funaba, Ph.D.
Division of Applied Biosciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan
Tel.: +81-75-753-6055
Fax: +81-75-753-6344
E-mail: mfunaba@kais.kyoto-u.ac.jp

Abstract

Expression of hepcidin, a central regulator of systemic iron metabolism, is transcriptionally regulated by the bone morphogenetic protein (BMP) pathway. However, the factors other than the BMP pathway also participate in the regulation of hepcidin expression. In the present study, we show that serum treatment increased hepcidin expression and transcription without inducing the phosphorylation of Smad1/5/8 in primary hepatocytes, HepG2 cells or Hepa1-6 cells. Co-treatment with LDN-193189, an inhibitor of the BMP type I receptor, abrogated this hepcidin induction. Reporter assays using mutated reporters revealed the involvement of the BMP response element-1 (BMP-RE1) and signal transducers and activator of transcription (STAT)- and activator protein (AP)-1-binding sites in serum-induced hepcidin transcription in HepG2 cells. Serum treatment induced the expression of the AP-1 components c-fos and junB in primary hepatocytes and HepG2 cells. Forced expression of c-fos or junB enhanced the response of hepcidin transcription to serum treatment. By contrast, expression of dominant negative (dn)-c-fos and dn-junB decreased hepcidin transcription. The present study reveals that serum contains factors stimulating hepcidin transcription. Basal BMP activity is essential for the serum-induced hepcidin transcription, although serum treatment does not stimulate the BMP pathway. The induction of c-fos and junB by serum treatment stimulates hepcidin transcription, through possibly cooperation with BMP-mediated signaling. Considering that AP-1 is induced by various stimuli, the present results suggest that hepcidin expression is regulated by more diverse factors than had been previously considered.

1. Introduction

Iron is essential for fundamental metabolic processes in cells and organisms. Iron homeostasis is strictly maintained through cellular and systemic systems. Cellular iron metabolism is regulated through iron-regulatory proteins that bind iron-responsive elements in regulated mRNAs. By contrast, systemic iron metabolism is mainly governed by the synthesis and secretion of the iron-regulating hormone hepcidin. Hepcidin is produced by liver parenchymal cells and orchestrates systemic iron fluxes and decreases plasma iron levels by binding to and degrading the iron exporter ferroportin on the surface of iron-releasing cells, especially on the basolateral membrane of enterocytes (Lee and Beutler, 2009; Hentze et al., 2010; Anderson et al., 2012).

Hepcidin expression is transcriptionally regulated; bone morphogenetic protein (BMP), a member of the transforming growth factor-β (TGF-β) family, potently stimulates the process (Muckenthaler, 2008; Lee and Beutler, 2009; Hentze et al., 2010; Finberg, 2013). Members of the TGF-β family, including BMP, TGF-β, and activin, elicit their activities through similar but distinct signal transduction pathways. Upon ligand binding, the receptor complexes phosphorylate carboxyl-terminal serines of receptor-regulated (R-) Smad; BMP induces the phosphorylation of Smad1/5/8, whereas TGF-β/activin do Smad2/3 phosphorylation. Phosphorylated R-Smad forms complexes with the common Smad, Smad4, and these translocate into the nucleus where they participate in the regulation of gene transcription (Miyazono et al., 2010; Massague, 2012; Sakaki-Yumoto et al., 2013).

In addition to the BMP pathway, several other molecules, such as interleukin (IL)-6, IL-22, oncostatin M and testosterone, also regulate hepcidin transcription (Nemeth et al., 2003; Kanda et al., 2009; Chung et al., 2010; Guo et al., 2013; Smith et al., 2013); other factors and mechanisms that regulate hepcidin expression have not yet been fully

elucidated.

During the exploration of BMP-induced hepcidin expression, we noticed that serum starvation down-regulates hepcidin expression in hepatocytes. This observation prompted us to pursue the role of serum as a positive regulator in hepcidin expression. Here we show that serum stimulates hepcidin transcription via the following: an activator protein (AP)-1 binding site within the hepcidin promoter, which spans nt -133 to nt -127 and was identified previously but was not characterized (Truksa et al., 2007); the signal transducers and activator of transcription (STAT)-binding site (STAT-BS), which spans nt -143 to nt -134; and the BMP response element 1 (BMP-RE1), which spans nt -155 to nt -150. Although serum treatment does not stimulate the BMP pathway, the basal BMP activity is indispensable for serum-induced hepcidin transcription. In addition, the AP-1 components c-fos and junB induced by serum treatment would be involved in the transcriptional activation. The present study extends our understanding of hepcidin expression. AP-1 is induced by various stimuli, such as stress, infections and inflammations, and certain stimuli that are mediated by cytokines and growth factors (Hess et al., 2004). Thus, the present results suggest that hepcidin is regulated by more factors than were previously considered.

2. Materials & methods

2.1. Materials

The following reagents were purchased and used: BMP2 was from R & D Systems (Minneapolis, MN, USA); BMP6 was from GeneTex (Irvine, CA, USA); LDN-193189, an inhibitor for BMP type I receptor (Yu et al., 2008a) was from Stemgent (San Diego, CA, USA); rabbit polyclonal antibodies against phospho-Smad1 (Ser463/Ser465) / Smad5 (Ser463/Ser465) / Smad8 (Ser426/Ser428) (#9511) and p38 (#9212) were from

Cell Signaling Technology (Danvers, MA, USA); a mouse monoclonal antibody against Smad1 (A-4, sc-7965) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); a rabbit monoclonal antibody against Smad1 (EP565Y, ab33902) and a mouse monoclonal antibody against β-actin (AC-15, ab6276) were from Abcam (Cambridge, MA, USA).

2.2. Cell culture

Animal experiments were approved by the Kyoto University Animal Experiment Committee. Primary rat hepatocytes were isolated by collagenase digestion of livers from male Sprague-Dawley rats aged 4 weeks. The livers were perfused from the portal vein to the incised inferior vena cava with calcium and magnesium-free (CMF) buffer (40 mM Hepes, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 5.0 mM $NaHCO_3$ and 5.6 mM glucose supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B) for 10 min at a rate of ~12 mL/min followed by perfusion with CMF buffer containing 0.05% collagenase (Wako, Osaka, Japan) for 10 min. Subsequently, hepatocytes were liberated into Hank's buffered salt solution (HBSS), i.e., 140 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 4.2 mM $NaHCO_3$ and 5.6 mM glucose supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B. After cell recovery by centrifugation at 50 × *g* for 2 min, the cells were washed with HBSS three times and were then resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Cells (>90% hepatocytes by microscopy) were seeded on collagen-coated plates at 1.5 $\times 10^5$ cells per well in 12-well plates. Attached cells were subsequently used. HepG2 hepatoma cells and Hepa1-6 hepatoma cells were cultured in DMEM with 10% heat-inactivated FBS and antibiotics.

2.3. RNA isolation and RT-quantitative PCR

Total RNA was isolated from rat primary hepatocytes by using TRIZOL (Invitrogen, Grand Island, NY, USA) and cDNA was synthesized by the ABI high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturers' protocols. The cDNA that was reverse-transcribed from 5 ng of total RNA was used as a template for RT-quantitative PCR (RT-qPCR) as previously described (Asano et al., 2013). The oligonucleotide primers for RT-qPCR are as follows: 5'-gatggcactcagcactgga-3' and 5'-gctgcagctctgtagtctgtct-3' for hepcidin, 5'-gaacactggatggacgactg-3' and 5'-acagacgggcatagatcaca-3' for Smad4, and 5'-cgtgttcctacccccaatgt-3' and 5'-tgtcatcatacttggcaggtttc-3' for glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The Ct value was determined, and the abundance of gene transcripts was analyzed using the ΔΔCt method and by normalizing against the Gapdh gene (Duran et al., 2005).

2.4. Western blot

Western blot analyses were performed as previously described (Funaba and Murakami, 2008). The immunoreactive proteins were visualized using the ECL Select Western blotting detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

2.5. Plasmids

The DNA sequence that spans nt -2018 to nt -35 of the mouse hepcidin (Hamp1) promoter was amplified and cloned into a pGL4 basic vector that contained the firefly luciferase reporter (Hepcidin (-2018)-luc); nt +1 was defined as the translation initiation site. Reporter plasmids with deleted promoters or point mutations were prepared by a PCR-based method. The dominant negative (dn)-c-fos, which lacks region encoding amino acid 133 to amino acid 159 (Ransone et al., 1990), was also prepared by a

PCR-based method. The plasmids were verified by DNA sequencing. Expression vectors for c-fos, junB and dn-jun B were kindly provided by Dr. M. Hibi.

2.6. Reporter assays

HepG2 and Hepa1-6 hepatoma cells were transiently transfected with the described expression vectors, reporter construct and a plasmid expressing β-galactosidase (pCMV-βGal) with the use of the polyethylenimine Max reagent (Polysciences, Warrington, PA, USA) according to the manufacturer's protocol. DNA transfection was performed in the absence of heat-inactivated FBS. Equal amounts of DNA, adjusted with empty vector, were transfected in each experiment. At 4 h post-transfection, cells were cultured with medium with or without heat-inactivated FBS for 24 h. Luciferase activity in the cell lysate was normalized to β-galactosidase activity.

2.7. Statistical analyses

Data are expressed as the mean ± standard error (SE). Data on gene expression were log-transformed to provide an approximation of a normal distribution before analysis. Differences between gene expression in the cells were examined using unpaired *t*-tests. Differences of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Serum stimulates hepcidin transcription in liver cells

When effects of exogenous ligand are examined, cells are typically cultured with a reduced concentration of heat-inactivated FBS before and during ligand stimulation. During the analysis of BMP-induced hepcidin expression in rat primary hepatocytes, we reproducibly observed that serum starvation itself down-regulates hepcidin expression significantly ($P < 0.05$, Fig. 1A). The decreased expression levels of hepcidin were not

caused by a non-specific down-regulation of gene expression, because serum starvation did not affect Smad4 expression. These results suggest that serum has a stimulatory effect on hepcidin expression. Thus, we aimed to verify the up-regulation of hepcidin expression levels in response to serum treatment. Attached rat primary hepatocytes were cultured in the absence of heat-inactivated FBS followed by treatment with or without 10% FBS. Consistent with the results on the effects of serum starvation (Fig. 1A), the serum treatment clearly increased expression of hepcidin but not Smad4 in primary hepatocytes ($P < 0.01$, Fig. 1B).

Previous studies revealed that hepcidin expression is primarily increased by activation of the BMP pathway (Lee and Beutler, 2009; Hentze et al., 2010; Finberg, 2013). Especially, BMP6 principally regulates hepcidin transcription in livers; mice lacking BMP6 exhibited inappropriately low level of hepcidin and massive iron overload (Andriopoulos et al., 2009; Meynard et al., 2009). BMP2 is relatively heat-stable, and heat-inactivation of FBS for 30 min at 56°C did not decrease the bioactivity of BMP2 (Ohta et al., 2005), although the stability of BMP6 in response to the heat treatment is unknown. Thus, it is possible that BMP or the related molecule(s) in heat-inactivated FBS is responsible for serum-induced hepcidin expression. To this end, we evaluated phosphorylated Smad1/5/8 in response to serum stimulation in primary hepatocytes because BMPs elicit their activities through the phosphorylation of carboxyl-terminal serines of Smad1/5/8 (Miyazono et al., 2010; Massague, 2012; Sakaki-Yumoto et al., 2013).

Serum treatment did not increase the phosphorylation levels of Smad1/5/8 in primary hepatocytes; rather, phosphorylated Smad1/5/8 levels tended to decrease with serum treatment, implying the presence of inhibitor(s) for the BMP pathway in serum (Fig. 1C, lanes 2-9). As expected, Smad1/5/8 was clearly phosphorylated within 1 h after BMP2

treatment in primary hepatocytes (Fig. 1C, lanes 10 and 11). Pre-treatment with LDN-193189 decreased phosphorylation levels of Smad1/5/8 (Fig. 1D) and abrogated serum-induced hepcidin expression (Fig. 1E).

To explore serum-induced hepcidin expression in detail, experiments were next performed in HepG2 cells and Hepa1-6 cells. Consistent with the results from primary hepatocytes, serum treatment did not induce the phosphorylation of Smad1/5/8 in either HepG2 cells or Hepa1-6 cells (Fig. 2A). We next examined hepcidin transcription by luciferase-based reporter assays in HepG2 cells and Hepa1-6 cells; these cell lines are suitable to examine hepcidin transcription but not hepcidin expression at the mRNA level (Kanamori et al., 2014). Serum treatment increased the expression levels of hepcidin(-2018)-luc in HepG2 cells and Hepa1-6 cells (Fig. 2B), which indicated that serum-induced hepcidin expression is regulated at the level of transcription. In addition, pre-treatment with LDN-193189 decreased luciferase activity and blocked serum-induced hepcidin transcription (Fig. 2B). These results indicate that BMP activity is not further enhanced by serum treatment but that endogenous BMP activity is required for serum-induced hepcidin expression and transcription in liver cells.

We also evaluated effects of BMP6 in heat-inactivated FBS on Smad1/5/8 phosphorylation and hepcidin transcription in HepG2 cells and Hepa1-6 cells. Herrera and Inman (2009) reported that FBS contains ~300 pM of BMP6, which was determined by transcriptional activity of BMP-responsive reporter. Thus, it is estimated that the culture medium, i.e., DMEM with 10% FBS, contains ~30 pM of BMP6 that was heated for 30 min for 56°C. Significant induction of Smad1/5/8 phosphorylation and hepcidin transcription could not be detected in HepG2 cells and Hepa1-6 cells treated with below 30 pM of the heat-treated BMP6 (Fig. 2C and D). Taken failure of Smad1/5/8 phosphorylation in response to serum treatment in primary hepatocytes (Fig.

1C and D) with the inability to phosphorylate Smad1/5/8 and transactivate hepcidin by 30 pM of the heat-treated BMP6 in live cell lines together, BMP in the FBS is not likely to explain serum-induced up-regulation of hepcidin expression. Previous studies also revealed that a BMP concentration greater than 100 pM is required to induce hepcidin expression (Truksa et al., 2006; Maes et al., 2010; Wu et al., 2012).

3.2. Serum-induced hepcidin transcription is mediated via BMP-RE1, TRE and STAT-BS

Previous studies revealed that the hepcidin promoter has two BMP responsive elements: BMP-RE1 (spanning nt -155 to nt -150) and BMP-RE2 (spanning nt -1678 to nt -1673) (Casanovas et al., 2009; Truksa et al., 2009). We performed reporter assays using a series of deleted reporters of mouse hepcidin promoter. The responsiveness to serum treatment was still detected by a deletion of BMP-RE2, although basal transcription was reduced and the extent of the increase in luciferase expression in response to serum treatment was decreased (Fig. 3A). The deletion of BMP-RE1 further decreased basal transcription and inhibited the responsiveness to serum treatment (Fig. 3A), which suggests an essential role of BMP-RE1 in serum-induced hepcidin transcription. Consistent with these results, reporter assays that used reporters with point mutations in BMP-RE1, BMP-RE2 or both revealed that serum-induced hepcidin transcription was inhibited in reporters containing mutations in BMP-RE1 but not BMP-RE2 (Fig. 3B).

Serum treatment is known to induce the expression of AP-1 components, which stimulate transcription by binding to the TPA-responsive element (TRE) located in the transcriptional regulatory region of the gene (Shaulian and Karin, 2002; Hess et al., 2004). The TRE consensus sequence is TGA$^{G}/_{C}$TCA (Shaulian and Karin, 2002; Hess et al., 2004), but the closely related sequence TGAGACA also binds to AP-1 and confers AP-1-mediated transcription (Kim et al., 1990). A search for the hepcidin gene

identified a possible TRE sequence from nt -133 to nt -127 (Fig. 4A).

Truksa et al. (2007) reported the existence of the TRE sequence in the hepcidin gene and explored its function in IL-6 and BMP9-induced hepcidin transcription by using a reporter plasmid containing the hepcidin promoter with TRE mutations. However, to create the "TRE mutated reporter" they mutated sequence outside of the core TRE sequence (TGA$^{G}/_{C}$TCA) (Truksa et al., 2007), and therefore the role of TRE was not strictly evaluated in their study. Additionally, adjacent to the TRE sequence, a STAT-BS sequence spans nt -143 to nt -134 (Fig. 4A). Although the STAT-BS sequence has been reported to function in IL-6-induced transcription (Wrighting and Andrews, 2006), mutations in the STAT-BS in mouse hepcidin (Hamp1) did not affect the responsiveness to IL-6 (Truksa et al., 2007). In the present study, we prepared reporter plasmids with mutated core TRE sequence (mTRE), STAT-BS sequence (mSTAT) or both (mSTAT, TRE) by introducing point mutations at distal sites from the mutual elements (Fig. 4B). In this case, we used a reporter plasmid with a shorter hepcidin promoter, hepcidin(-270)-luc, as a wild-type plasmid to evaluate serum responsiveness (Fig. 3A).

Serum-induced hepcidin transcriptional activation was decreased when the TRE or STAT-BS was mutated (Fig. 4C), and the double mutations completely abrogated serum-induced hepcidin transcription. In addition, mutations in the TRE decreased basal transcription levels of hepcidin. These results suggest that both the TRE and STAT-BS are required for full activation of hepcidin transcription induced by serum; especially, TRE is essential for the basal transcription of hepcidin.

Verga Falzacappa et al. (2007) identified a possible AP-1 binding site but not the TRE in the human hepcidin gene, which extends from nt -313 to nt -304, by use of the nucleotide database on putative transcription factor binding sites. The nucleotide

database did not indicate the corresponding region of mouse hepcidin gene (Hamp1), nt -292 to nt -283, as a putative AP-1 binding site (Fig. 5A). Nevertheless, we examined the role of the region spanning from nt -292 to nt -283 by use of hepcidin(-581)-luc as a wild-type plasmid. The responsiveness to serum was not changed by the mutations of the region (Fig. 5B). The results are consistent with those that deletion of nt -581 to nt 271 did not affect hepcidin transcription in response to serum (Fig. 3A). Thus, we conclude that the putative AP-1 site suggested by Verga Falzacappa et al. (2007) is not functional for serum-induced transcription of mouse hepcidin.

3.3. Serum induces c-fos and junB expression in liver cells, which is responsible for hepcidin transcription via TRE and STAT-BS

We examined the induction of AP-1 in response to serum treatment in primary hepatocytes (Fig. 6A) and HepG2 cells (Fig. 6B). The time-course changes that occurred during serum stimulation were similar between the two cell types but were different among the genes analyzed; c-fos expression was transiently induced within 30 min after serum treatment, and c-fos expression levels returned to basal levels at 1.5 h after serum treatment in primary hepatocytes and at 2 h in HepG2 cells. Expression levels of junB significantly higher in cells treated with serum at 1 h of serum treatment in primary hepatocytes and from 0.5 h to 4 h in HepG2 cells. In contrast, serum treatment did not significantly affect c-jun expression in primary hepatocytes, although it slightly but significantly increased from 0.5 to 4 h in HepG2 cells.

Considering that serum treatment stimulated hepcidin expression and transcription in primary hepatocytes and HepG2 cells, expression of c-fos and junB but not c-jun may be involved in serum-induced hepcidin transcription. Thus, we explored the effects of the forced expression of c-fos and junB were examined (Fig. 7). Expression of c-fos alone increased transcription of hepcidin, and it also increased serum-induced hepcidin

transcription. The responsiveness to serum also increased with junB expression, although junB expression alone did not stimulate hepcidin transcription. Mutations of either the STAT-BS or TRE sequences decreased hepcidin transcription, irrespective of serum stimulation. The effects of the TRE mutations were more remarkable, but the reporters still responded to serum treatment. Double mutations in both the STAT-BS and TRE sequences completely inhibited hepcidin transcription.

We further examined effects of expression of dn-c-fos or dn-junB on hepcidin transcription (Fig. 8). Basal transcription of hepcidin was decreased by expression of dn-c-fos or dn-junB. In addition, the extent of the increase in luciferase activity in response to serum treatment was smaller in cells expressing dn-c-fos or dn-junB than in those expressing empty vector.

Here, we showed that withdrawal of heat-inactivated FBS decreased the expression of hepcidin in primary hepatocytes and that serum treatment increased hepcidin expression and transcription (and vice versa) in primary hepatocytes, HepG2 cells and Hepa1-6 cells. We also demonstrated that serum treatment does not stimulate the BMP pathway but basal BMP activity is required for hepcidin induction through the BMP-RE1 region of its promoter. The serum induced expression of c-fos and junB, and both the TRE and STAT-BS sequences within the hepcidin promoter are essential for serum-induced transcription. AP-1 enhances the transcription of the α2-macroglobulin gene through complex formation with STAT3 (Ginsberg et al., 2007). The requirement of adjacent STAT-BS and TRE sequences suggests the cooperative role of AP-1 and STAT3 in AP-1-mediated hepcidin transcription, similar to that for the α2-macroglobulin gene.

Hepcidin transcription, via both STAT-BS and TRE regulation, may be elicited by some cytokines. For example, oncostatin M, a stimulator of hepcidin expression (Kanda et al.,

2009; Chung et al., 2010), can induce the expression of AP-1 components (Botelho et al., 1998). Not only JAK inhibition but also MEK1/2 inhibition blocked oncostatin M-mediated hepcidin expression (Kanda et al., 2009; Chung et al., 2010); JAK phosphorylates and activates STAT (Ihre, 2001), and MEK1/2 is a MAPK kinase and an upstream molecule that induces AP-1 components (Shaulian and Karin, 2002).

The present study reveals the requirements of BMP-RE1, TRE and STAT-BS for the full induction of hepcidin transcription by serum treatment; BMP-RE1 is essential for the responsiveness to serum, although BMP activity is not enhanced by serum. In view of a central role of the BMP pathway in hepcidin transcription, AP-1 possibly accelerates hepcidin transcription through enhancement of basal BMP activity. Previous studies revealed AP-1 cooperatively enhanced TGF-β-mediated transcription through activated Smad3 and Smad4 (Zhang et al., 1998; Wong et al., 1999; Funaba et al., 2006; Busnadiego et al., 2013), but at present, no direct evidence is available on the role of AP-1 in BMP-mediated signaling. Members of the Jun family physically interact with Smad4 (Liberati et al., 1999), a common Smad that transmits both TGF-β and BMP signals (Miyazono et al., 2010; Massague, 2012; Sakaki-Yumoto et al., 2013). In addition, a liver-specific disruption of Smad4 decreased expression of hepcidin in mice, and overexpression of Smad4 increased transcription of hepcidin in Hepa1-6 cells (Wang et al., 2005), which indicates the involvement of Smad4 in hepcidin expression. To clarify the role of AP-1 in BMP signaling, the effects of exogenous BMP should be examined in future studies.

During the preparation of this manuscript, the effect of serum treatment on hepcidin transcription was reported (Shanmugam and Cherayil, 2013). These researchers showed that heat-inactivated FBS treatment increased the phosphorylation levels of Smad1/5/8 and hepcidin mRNA levels in HepG2 cells, which were blocked by co-treatment with

dorsomorphin, an inhibitor of the BMP type I receptor, as well as AMPK (Zhou et al., 2001; Yu et al., 2008b). These authors concluded that BMP or BMP-related proteins in heat-inactivated FBS are responsible for the serum-induced hepcidin mRNA changes, and these researchers' results on serum-induced hepcidin expression are consistent with those of the present study. However, the underlying mechanism clearly contrasts with our results; the reason for this inconsistency is currently unknown.

Acknowledgments

We thank Dr. M. Hibi for providing plasmids. This work was partly supported by a research project grant that was awarded by the Azabu University.

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Figure legend

Fig. 1. Modulation of hepcidin expression levels by serum concentrations in rat primary hepatocytes

(A) Attached rat primary hepatocytes were cultured in the presence or absence of heat-inactivated FBS for 12 h. Hepcidin expression was examined by RT-qPCR and expressed as ratios to Gapdh, with the level set to 1 in cells cultured in the presence of heat-inactivated FBS. Data shown are the mean ± SE (n = 3). *: $P < 0.05$, vs. cells treated with 10% FBS. (B) Attached rat primary hepatocytes were cultured in the absence of heat-inactivated FBS for 4 h, followed by culturing with or without 10% heat-inactivated FBS for 12 h. Hepcidin expression was examined by RT-qPCR and is expressed as ratios to Gapdh, with the level set to 1 in cells treated without FBS. Data shown are the mean ± SE (n = 4). **: $P < 0.01$, vs. cells treated without serum. (C) Attached rat primary hepatocytes were cultured in the presence or absence of heat-inactivated FBS for the indicated time. Phosphorylated Smad1/5/8, Smad1 and p38 were examined by Western blot analysis. As a positive control, rat primary hepatocytes were also treated with BMP2 (4 nM) for 1 h. (D and E) Attached rat primary hepatocytes were treated with or without LDN-193189 for 20 min followed by treatment with or without 10% heat-inactivated FBS for 1 h (D) or 12 h (E). (D) Phosphorylated Smad1/5/8, Smad1 and p38 were examined by Western blot analysis. (E) Hepcidin expression was examined by RT-qPCR and is expressed as ratios to Gapdh, with the level set to 1 in cells treated without LDN-193189 and FBS. Data shown are the mean ± SE (n = 4). **: $P < 0.01$, vs. cells treated with the respective reagent (vehicle or LDN-193189) but without serum. ‡: $P < 0.01$, vs. cells treated with vehicle and serum.

Fig. 2. Role of endogenous BMP activity in serum-induced hepcidin expression

(A and C) HepG2 cells and Hepa1-6 cells were cultured in the absence of

heat-inactivated FBS for 4 h. (A) After pre-treatment with or without LDN-193189 for 20 min, cells were cultured with or without 10% heat-inactivated FBS for 1 h. (C) Cells were treated with or without various concentration of BMP6, which was heated for 30 min at 56°C, for 1 h. Concentration of the treated BMP6 is as follows; lanes 2 and 9: 0.24 pM, lanes 3 and 10: 1.2 pM, lanes 4 and 11: 6 pM, lanes 5 and 12: 30 pM, lanes 6 and 13: 150 pM. As a positive control, cells were treated with 4 nM BMP6 (lanes 7 and 14). (A and C) Phosphorylated Smad1/5/8, Smad1 and β-actin were examined by Western blot analysis. (B and D) After transfection with Hepcidin(-2018)-luc and CMV-βGal, HepG2 cells and Hepa1-6 cells were cultured in the absence of heat-inactivated FBS for 4 h. (B) After pre-treatment with or without LDN-193189 for 20 min, cells were cultured with or without 10% heat-inactivated FBS for 24 h. (D) Cells were treated with or without 30 pM BMP6, which was heated for 30 min at 56°C, for 24 h. As a positive control, cells were treated with 4 nM BMP6. (B and D) Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity in cells cultured in the absence of LDN-193189 and FBS was set to 1. Data were expressed as the mean ± SE from a representative experiment (n = 3).

Fig. 3. Role of BMP-RE in serum-induced hepcidin expression

After transfection with the indicated reporter plasmid and CMV-βGal, HepG2 cells were cultured in the absence of heat-inactivated FBS for 4 h. After pre-treatment with or without LDN-193189 for 20 min, cells were cultured with or without 10% heat-inactivated FBS for 24 h. Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity in cells that were cultured in the absence of FBS and were transfected with hepcidin(-2018)-luc was set to 1. Data were expressed as the mean ± SE from a representative experiment (n = 3). Hepcidin transcription was examined by the use of reporter genes with series of deletions (A) or a reporter gene with point mutations in BMP-RE1 and -RE2 (B). BMP-RE1 (GGCGCC) was mutated

to aGaaCC, and BMP-RE2 (GGCGCC) was mutated to tcaGCC; the mutated nucleotides are shown in small characters.

Fig. 4. TRE in the hepcidin promoter

(A) Nucleotide sequence of the hepcidin promoter around TRE. Possible TRE sequence is indicated by a box, and BMP-RE1 and STAT-BS are indicated by a double underline and a dotted underline, respectively. (B) Mutations in STAT-BS and TRE. TRE and STAT-BS are indicated by a box and a dotted underline, respectively. The mutated nucleotides are shown in small characters. (C) The role of TRE and STAT-BS in serum-induced hepcidin transcription. After transfection with the indicated reporter plasmid and CMV-βGal, HepG2 cells were cultured in the absence of heat-inactivated FBS for 4 h followed by culture with or without 10% heat-inactivated FBS for 24 h. Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity in cells that were cultured in the absence of FBS and were transfected with hepcidin(-270)-luc was set to 1. Data were expressed as the mean ± SE from a representative experiment (n = 3).

Fig. 5. Role of a putative AP-1 binding site in serum-induced hepcidin expression

(A) Nucleotide sequence of human putative AP-1 binding site and the corresponding region in mouse and rat hepcidin promoter. (B) The role of the putative AP-1 binding site in serum-induced hepcidin transcription. After transfection with the indicated reporter plasmid and CMV-βGal, HepG2 cells were cultured in the absence of heat-inactivated FBS for 4 h followed by culture with or without 10% heat-inactivated FBS for 24 h. Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity in cells that were cultured in the absence of FBS and were transfected with hepcidin(-581)-luc was set to 1. Data were expressed as the mean ± SE from a representative experiment (n = 3). The corresponding region to human putative AP-1

binding site (TGTGACATCA) was mutated to TGTGttATCA; the mutated nucleotides are shown in small characters.

Fig. 6. The induction of AP-1 components in response to serum stimulation

Primary hepatocytes (A) and HepG2 cells (B) were cultured in the absence of heat-inactivated FBS for 4 h followed by culturing with or without 10% heat-inactivated FBS for the indicated time. Expression of AP-1 components such as c-fos, c-jun and junB was examined by RT-qPCR and expressed as ratios to Gapdh, with the level in cells at time = 0 set to 1. Data shown are the mean ± SE (n = 4). * and **: $P < 0.05$ and $P < 0.01$, respectively, vs. cells treated without serum at the same time point.

Fig. 7. Enhancement of hepcidin transcription by AP-1 via TRE

The role of AP-1 components in serum-induced hepcidin transcription. After transfection with the indicated AP-1 component expression vector and reporter plasmid, and CMV-βGal, HepG2 cells were cultured in the absence of heat-inactivated FBS for 4 h followed by culturing with or without 10% heat-inactivated FBS for 24 h. Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity was set to 1 in cells that were cultured in the absence of exogenous AP-1 components and FBS and were transfected with hepcidin(-270)-luc. Data were expressed as the mean ± SE from a representative experiment (n = 3).

Fig. 8. Inhibition of hepcidin transcription by dn-c-fos and dn-junB

After transfection with the indicated dominant negative form of the AP-1 component expression vector and reporter plasmid, and CMV-βGal, HepG2 cells were cultured in the absence of heat-inactivated FBS for 4 h followed by culturing with or without 10% heat-inactivated FBS for 24 h. Luciferase activity was normalized to β-galactosidase activity, and the luciferase activity was set to 1 in cells that were cultured in the absence

of exogenous AP-1 components and FBS and were transfected with hepcidin(-270)-luc. Data were expressed as the mean ± SE from a representative experiment (n = 3).

Fig. 1

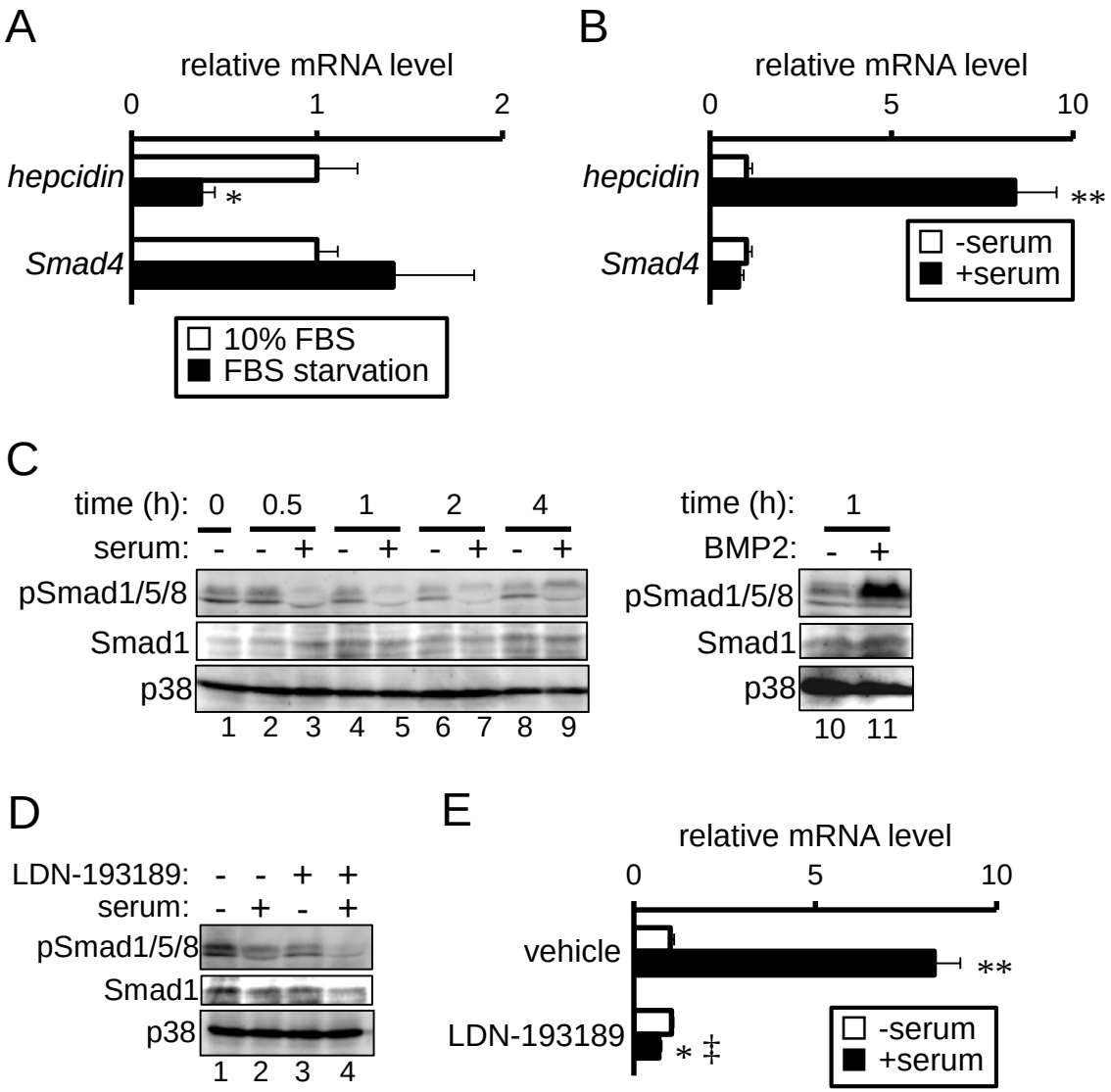

Fig. 2

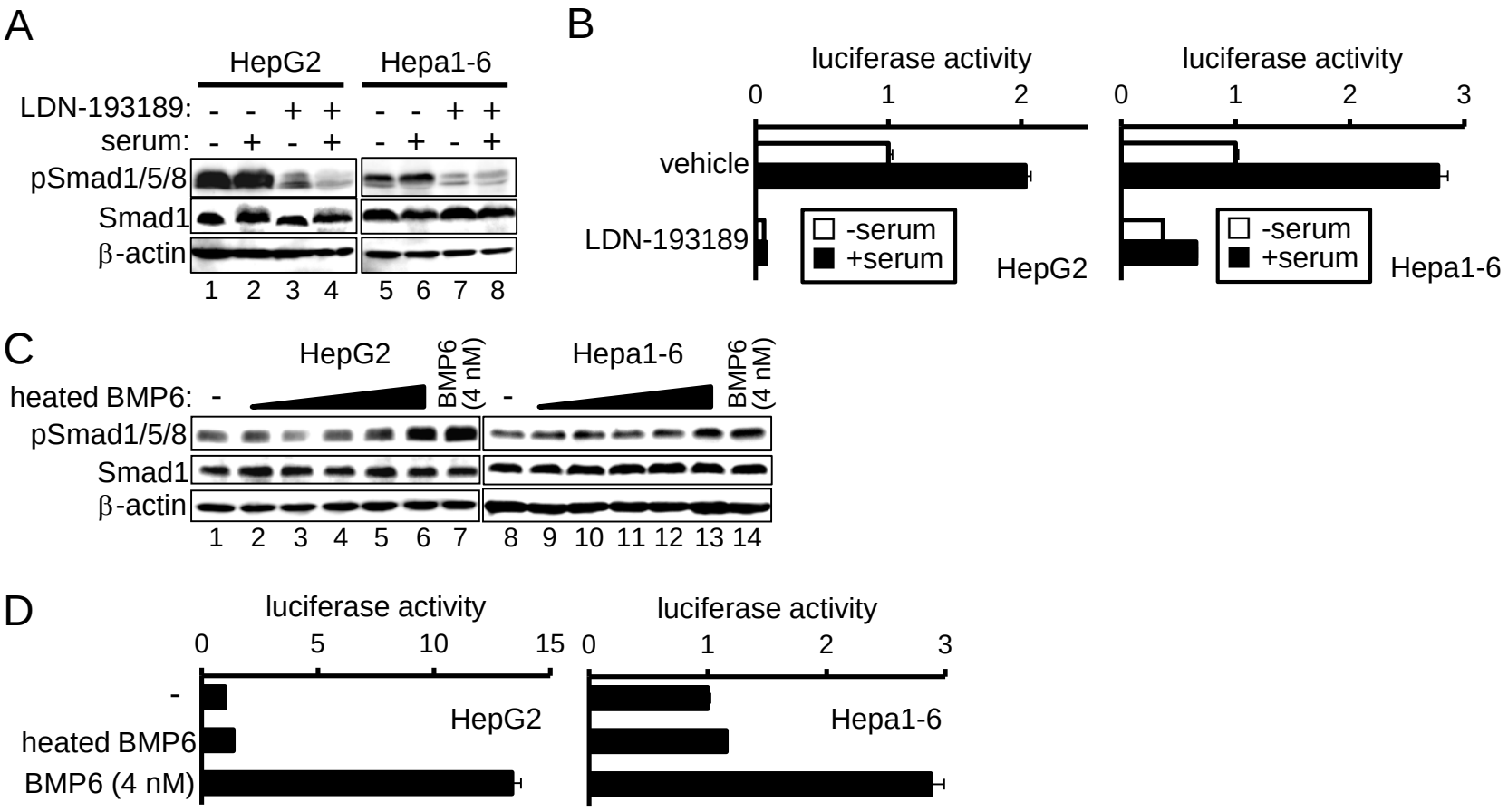

Fig. 3

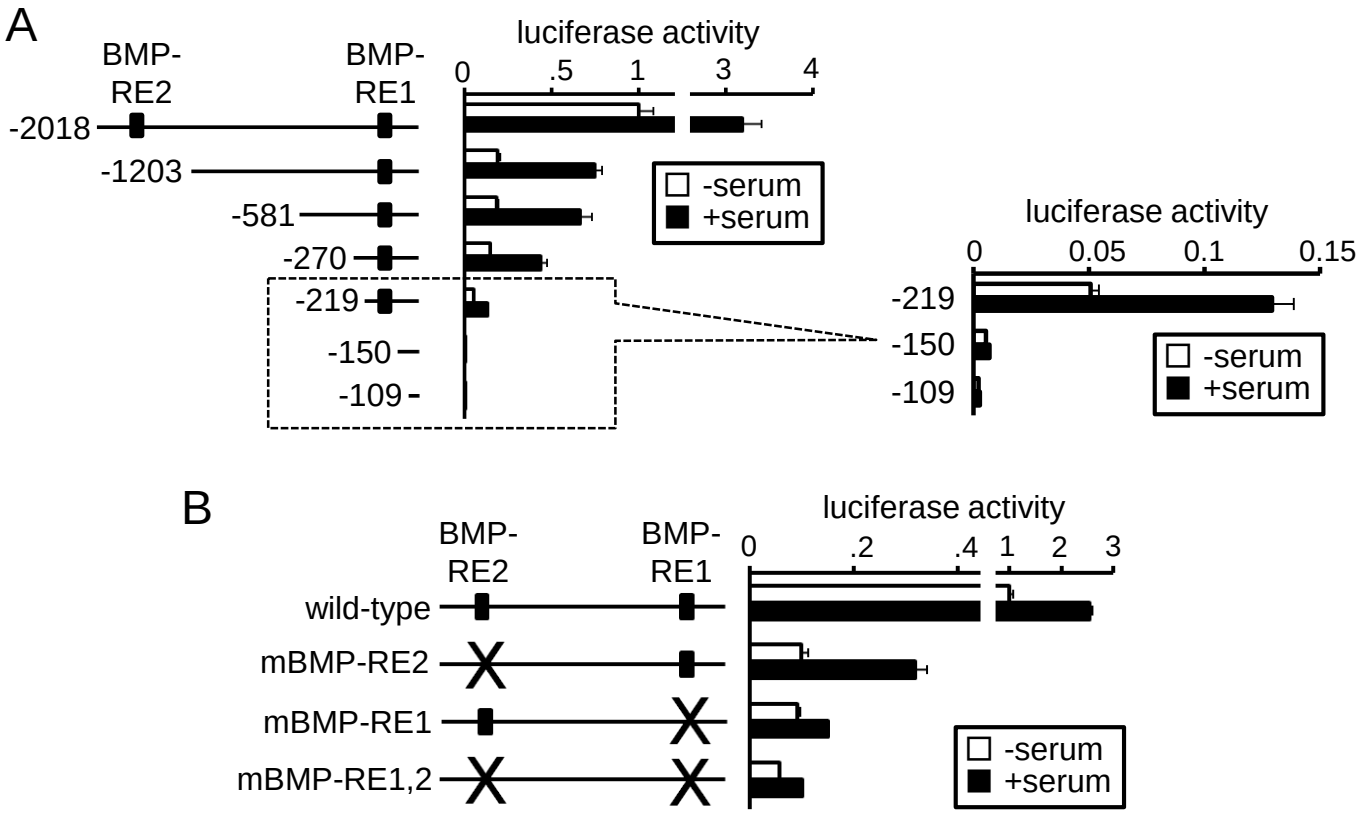

Fig. 4

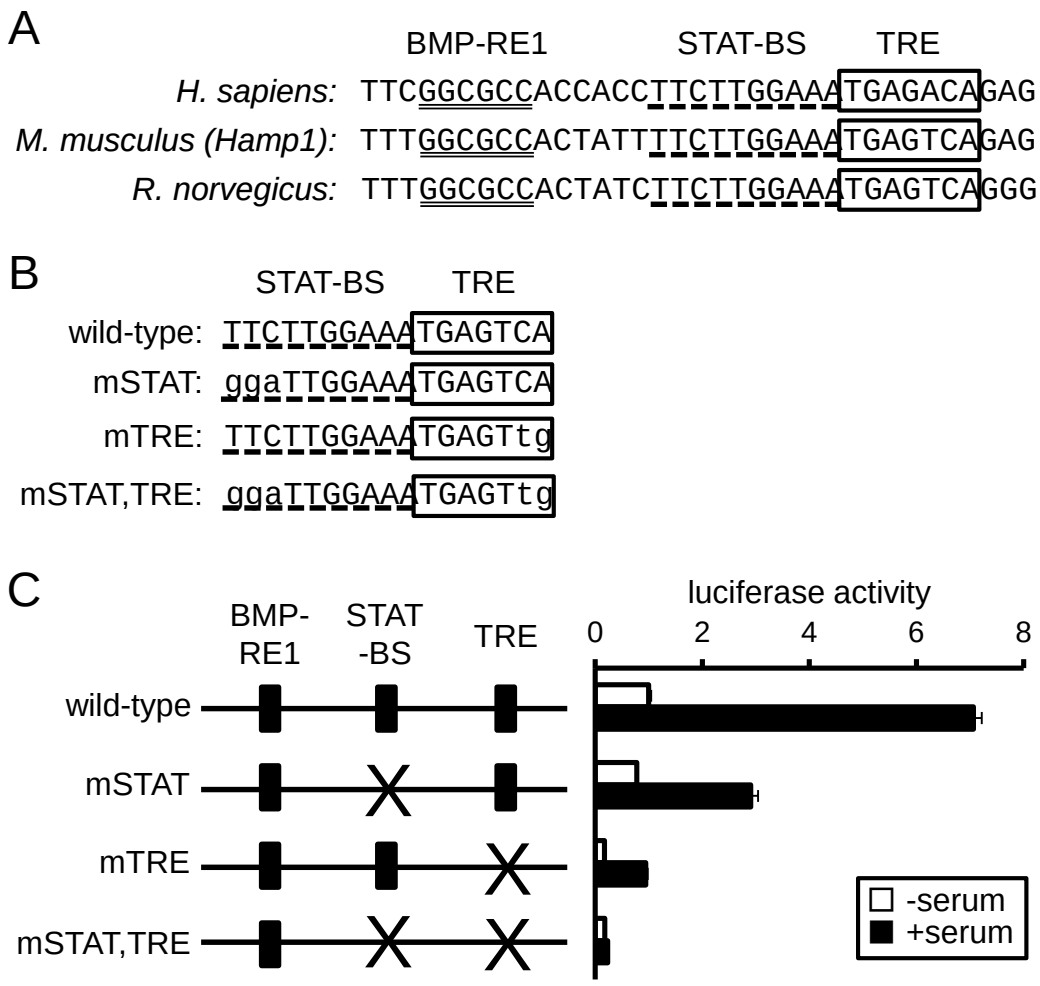

Fig. 5

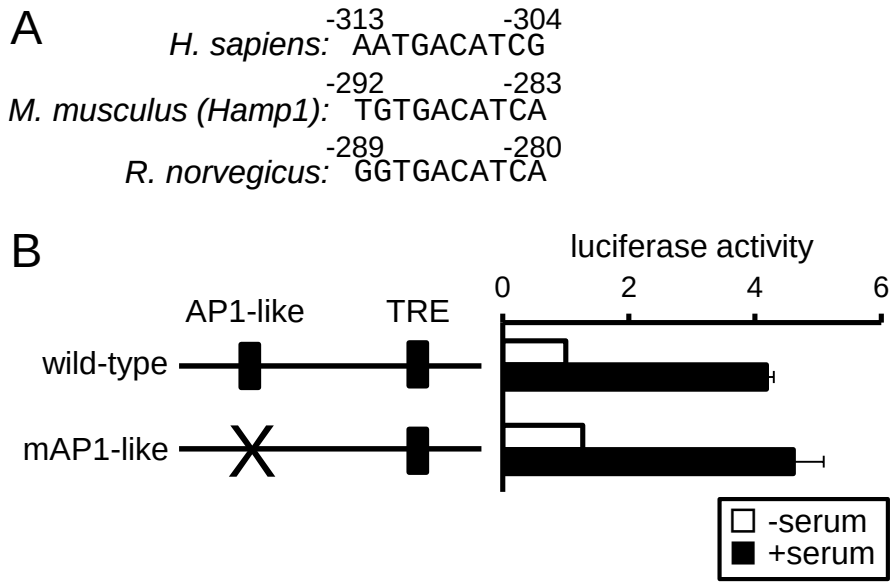

Fig. 6

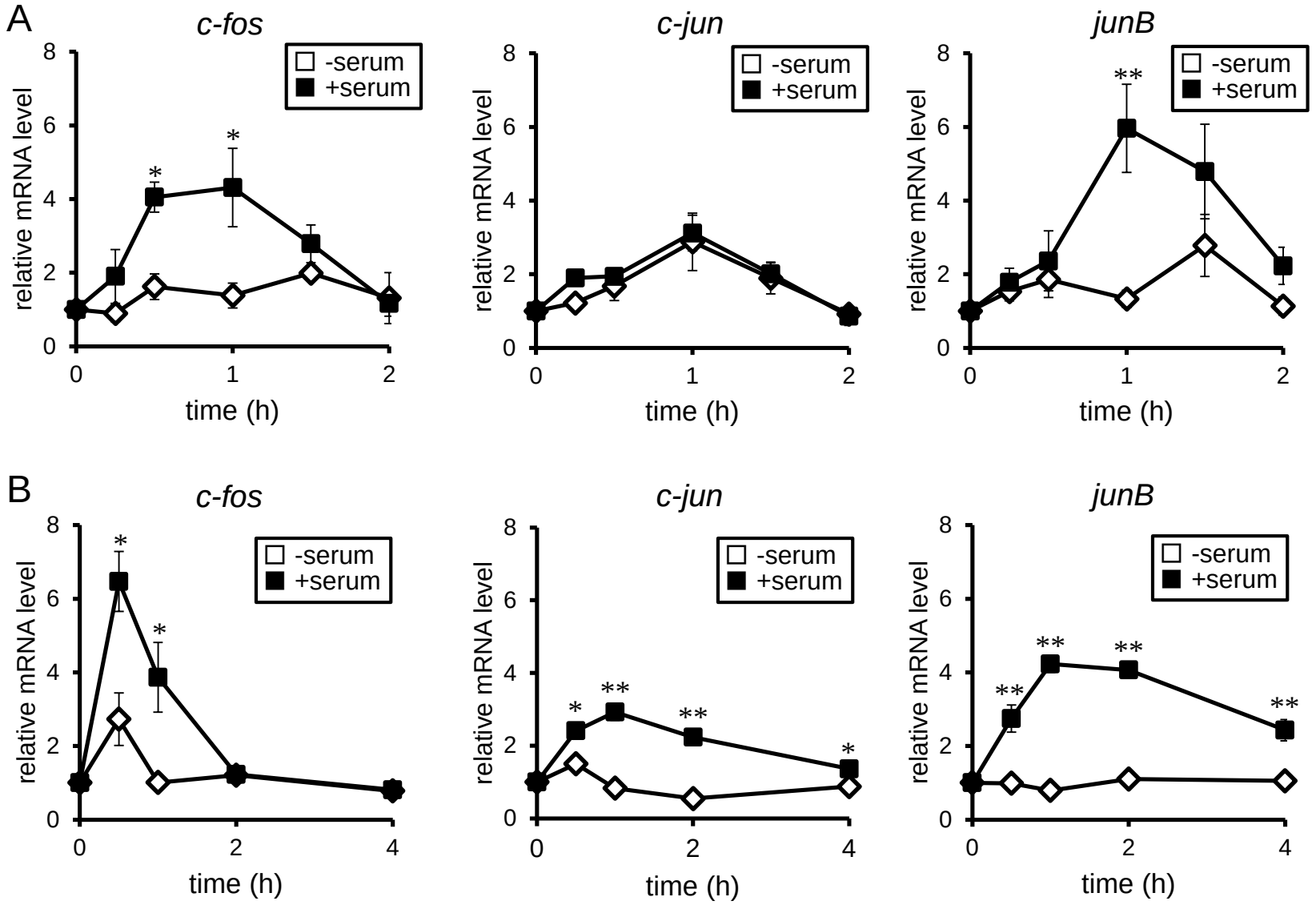

Fig. 7

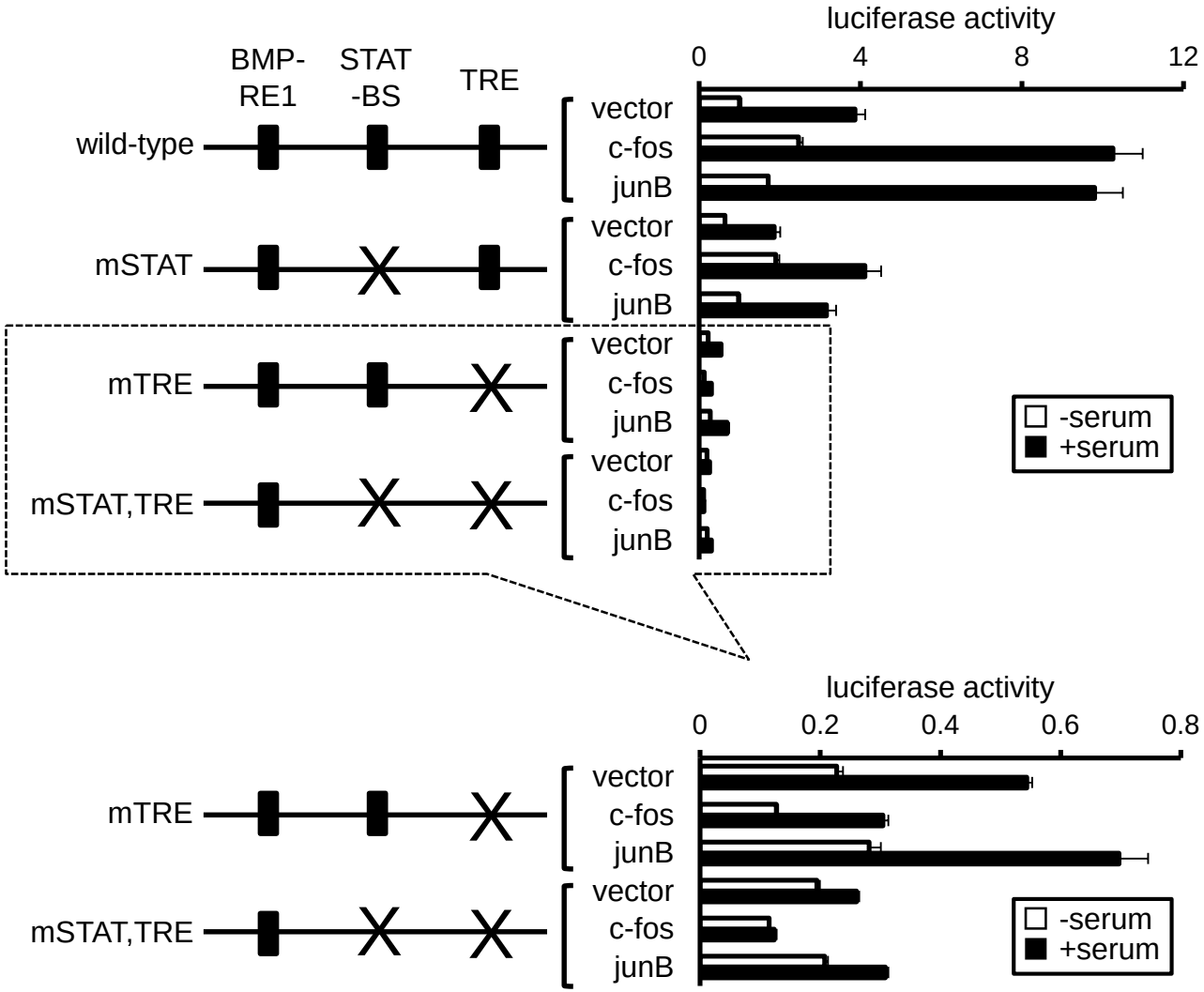

Fig. 8